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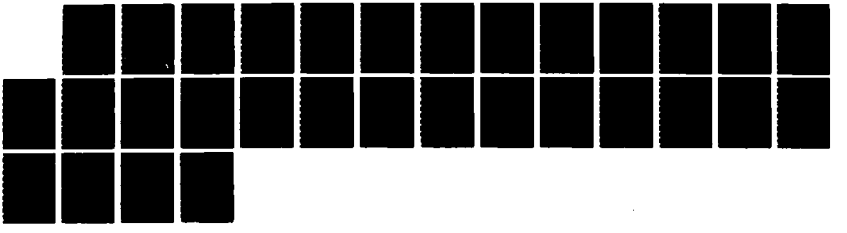

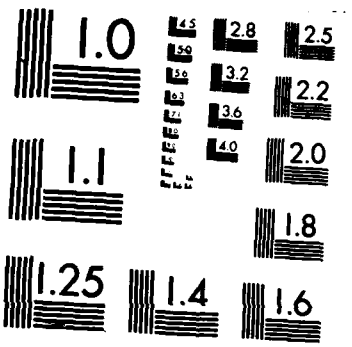

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AUTOMATED SAMPLE DEOXYGENATION FOR IMPROVED LUMINESCENCE MEASUREMENTS

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BRIEF

An automated sample deoxygenation system has been developed and demonstrated using a variety of polynuclear aromatic compounds. The deoxygenation procedure has an average relative standard deviation of < 4% of the observed luminescence intensity and a deoxygenation efficiency approaching 99% for cyclohexane systems.

ABSTPACT

Quenching of luminescence by molecular oxygen is an inherent limitation in fluorescence and phosphorescence analysis. Consequently, greater analytical sensitivity, as well as lower detection limits, can be achieved by removal of oxygen from the sample prior to luminescence analysis. An automated system for rapid deoxygenation is described in this paper. This apparatus combines a multiple sampling valve and a membrane barrier to generate a concentration gradient for sample deoxygenation. This automatic, multisample, on-line deoxygenation technique reduces sample handling to a minimum because deoxygenation and luminescence analysis occur within a closed system. The usefulness of this procedure is discussed in terms of automation, reproducibility, detection limit, deoxygenation efficiency and applications to a variety of compounds. In addition, potential applications of this procedure to other analytical subdisciplines is also discussed.

INTRODUCTION

Luminescence spectroscopy has become a widely used analytical method because of its low detection limits and inherent selectivity (1,2). In recent years, multidimensional luminescence analysis (3-5) and the development of rapid scanning fluorometers (6,7) have augmented the role of luminescence measurement as an analytical procedure. Selective quenching also provides an option in luminescence analysis. Since all compounds are not equally sensitive to the same quenchers, the luminescence of some unwanted interferents can be eliminated by careful selection of an appropriate quencher (8). Conversely, one of the most serious limitations to luminescence analysis also results from quenching. In this case, we refer to quenching by molecular oxygen. A large majority of fluorescence measurements and all solution phosphorescence measurements at room temperature are adversely affected by the presence of oxygen, because most organic molecules in an excited state will be dynamically quenched after one or two collisions with molecular oxygen. The interactions by which oxygen quenches luminescence have received considerable attention and thus, have been the subject of many investigations (9-12). These interactions have not been clearly defined and, therefore, several plausible mechanisms have been proposed (9-12). However, regardless of the mechanism of the process, oxygen quenching significantly limits luminescence analysis of many long lived fluorophores and of all phosphors. Therefore, oxygen should ideally be removed from the analyte matrix prior to experimentation. However, since oxygen is ubiquitous, its removal and exclusion from the analyte matrix is a challenging task. Consequently, a variety of sample deoxygenation methods have been developed. These methods include nitrogen (13) or argon purging (14), evacuation (15), freeze-pump-thaw cycles (16), ultrasonification (17), chemical scavenging methods (18) and the use of semipermeable membranes for

continuous oxygen removal (19). Each of these methods has limitations with respect to ease of implementation, amount of required sample handling, time constraints of the method and/or deoxygenation efficiency. Therefore, the investigation and improvement of sample deoxygenation methodology is ongoing.

This paper describes the automation of our previously reported deoxygenation apparatus (20). The theory of the deoxygenation process has been explained and its feasibility has been previously demonstrated (20). The analytical usefulness of this technique will be discussed in this manuscript in terms of automation, reproducibility, detection limit, deoxygenation efficiency, applicability to long-lived luminescent species and potential applications to other analytical subdisciplines.

THEORY

Basic Principles. Our deoxygenation procedure is based on membrane permeability and the ability to generate a concentration gradient. The membrane was chosen to satisfy two criteria. The first criterion was that it must have a high permeability to oxygen, and the second was that it be inert to organic solvents. The objective of the procedure is to minimize the concentration of oxygen in the sample. This occurs via oxygen diffusion (1) from the region of high concentration, i.e., the sample, (2) to the membrane surface, (3) through the membrane and (4) to the region of low concentration, i.e., a scavenging solution. The oxygen scavenging solution immediately consumes molecular oxygen and chemically reduces it to water. Thus, oxygen continues to diffuse through the membrane to reduce the concentration difference across the membrane until the sample is essentially oxygen free. This hypothesis assumes that (1) diffusion through the membrane is rapid, (2) sufficient time is allowed for diffusion of oxygen

through the membrane and (3) the scavenging reaction proceeds far enough to the right such that the concentration of oxygen is negligibly small at equilibrium.

Solubility and Membrane Permeability. The efficiency of the deoxygenation apparatus per unit time depends on the initial concentration of dissolved oxygen in the sample as well as the rate of oxygen diffusion through the membrane. These parameters can be described by Henry's Law, and Fick's First and Second Laws. Interested readers may consult any of several texts for a detailed explanation of the theories of solubility and diffusion as governed by these laws (21, 22).

Oxygen Quenching. Collisional quenching of fluorescence emission is described by the Stern-Volmer equation. For the specific case of quenching of fluorescence by molecular oxygen, the equation may be represented as shown in equation 1.

$$\frac{I_o}{I_{O_2}} = K_{sv}[O_2] = k_{O_2}\tau_o[O_2] \qquad (1)$$

The ratio of the fluorescence intensities of the species of interest in the absence and the presence of oxygen, respectively, which we refer to as the enhancement factor, is represented by the quantity I_o/I_{O_2}. The Stern-Volmer constant for collisional quenching is represented by K_{sv}. The bimolecular quenching constant is denoted as k_{O_2}. The lifetime of the fluorophore in the absence of oxygen is represented as τ_o. This relationship can also be used to predict the effect of various fluorescence lifetimes on the enhancement factor. The enhancement factor, EF, can be substituted directly into equation 1 to yield equation 2.

$$EF = \frac{I_{maximum}}{I_{O_2}} = k_{O_2}\tau_o[O_2] \qquad (2)$$

Thus, a plot of enhancement factor, EF, versus lifetime, τ_o, should produce a linear relationship with slope equal to $k_{O_2}[O_2]$. From this relationship one may deduce that as the lifetime of the fluorophore increases so will the potential enhancement factor, since the two parameters are directly related. Accordingly, as the lifetime of the fluorophore increases so does its susceptibility to oxygen quenching. Thus, sample deoxygenation is expected to be increasingly important as the luminescence lifetime of the fluorophore increases.

EXPERIMENTAL SECTION

Deoxygenation Apparatus. A schematic representation of the deoxygenation apparatus is shown in Figure 1. This apparatus is a completely automated, miniature version of our previously described apparatus (20). Pumping of the sample is controlled by two Gilson Minipuls 2 peristaltic pumps (Gilson Medical Electronics, Inc.; Middleton, WI), each of which regulates six of the twelve channels of solvent flow. Typical solvent flow rates of 0.3 to 0.5 ml/min were used. The sampling valve was obtained from Scanivalve Corporation (San Diego, CA). The valve is solenoid actuated and contains one output tube for the selected sample, plus another twelve tubes for the eleven unsampled streams to flow through the valve so that each stream is always updated to contain a fresh sample. A stream switching valve (Rainin Instrument Company; Woburn, MA) is used, when necessary, to bypass the deoxygenator and divert sample flow directly from the sampling valve to the spectrophotometer. A solenoid air valve (Rainin Instrument Company) and two 4-way pneumatic actuated slider valves (Rainin Instrument Company) are used to inject the sample into the solvent stream. Deoxygenation of the sample is achieved by generation of a concentration gradient across a 0.2 mil very low density polyethylene (VLDP)

membrane (Union Carbide Corporation; Bound Brook, NJ), that is mounted in a 12-inch dialyzer assembly (Technicon Industrial Systems; Tarrytown, NJ), as shown in Figure 2. A 0.1M acidic chromium (II) solution, generated in the presence of amalgamated zinc, is used as the oxygen scavenging agent. Oxygen exchange occurs across the flow channel of dimensions 12" x 1/16" x 1/25". As oxygen diffuses from the sample, through the membrane and into the scavenging solution, it is consumed according to the following reactions.

$$4Cr^{+2} + O_2 + 4H_3O^+ \longrightarrow 4Cr^{+3} + 6H_2O \qquad (3)$$

$$2Cr^{+3} + Zn(Hg) \longrightarrow 2Cr^{+2} + Zn^{+2} + Hg \qquad (4)$$

The chromium (II) solution is pumped continuously through the dialyzer using a Masterflex Pump System (Cole-Parmer Instrument Company; Chicago, IL). Following deoxygenation of the sample, the luminescence intensity is monitored with a Perkin-Elmer LS-5 fluorescence spectrophotometer; after which the sample is transported to waste and a fresh sample is analyzed via the same procedure. This process may be repeated as many times as necessary, until all samples have been analyzed.

Automation. All of the electronic hardware necessary for automation of the deoxygenation apparatus was developed and produced in our laboratory. Advancement of the sampling valve, timing of the pumps, and thus, regulation of the amount of deoxygenation time, regulation of the stream switching valve and of the sample injection valve are all controlled from the spectrophotometer. The Perkin-Elmer 3600 Data Station, used for computer control in this study, does not have an external access port which would permit direct link of the electronic hardware of the deoxygenation apparatus to the computer. Therefore, a control signal had to be generated by using one of the display LEDs of the spectrophotometer. The excitation monochromator LED of the LS-5 was chosen for this purpose. Any other LED

could have been selected for the same purpose. After proper amplification and conditioning, a standard TTL signal is obtained. This represents only a single bit of information; therefore, sorting is necessary in order to control all of the necessary functions. This is achieved by using a 4-bit counter (SN74LS93) and a 3 to 8 decoder circuit (SN74LS138). One can increase the number of devices being controlled by replacing this decoder. An appropriate feed back resets the circuit after the desired number of steps. The operation of the circuit is summarized by the flowchart shown in Figure 3. Acquisition of the deoxygenation data is achieved via computer control of the LS-5 spectrophotometer using the Perkin-Elmer 3600 data station. The data is stored on mini-floppy disk by the data acquisition program for later retrieval and processing.

Evaluation of Injection System. The components of the injection system include a solenoid air valve, a pneumatic slider injection valve and a 100 μL sample loop. The reproducibility and dispersion characteristics of the components were evaluated in the following manner. Twelve 100 μL samples of 4×10^{-5}M pyrene (99+% purity, Aldrich Chemical Company) in cyclohexane (Burdick and Jackson) were automatically injected into the sample deoxygenator. The average relative standard deviation of the injection system was calculated. The dispersion coefficient of the sample plug due to injection and deoxygenation was also calculated.

Pyrene Calibration Experiments. Pyrene in cyclohexane at five concentrations was used in these experiments. All compounds were used as received without further purification. A sample from each stream of the sampling valve was deoxygenated for 5 minutes and fluorimetrically analyzed at λ_{ex} = 336nm and λ_{em} = 383nm. This procedure completed one cycle of each experiment. Thus, one cycle was made up of twelve analyses of deoxygenated

pyrene solution at one of the concentrations noted. Each cycle was repeated twice, for a total of twenty-four deoxygenated samples per concentration of pyrene solution. The data from each experiment was used to judge the reproducibility and precision of the deoxygenation apparatus. Establishment of the linearity of response of the apparatus was necessary because the possibility of some non-linearity due to the fixed deoxygenation time was a concern.

Oxygen Calibration Experiments. Experiments to determine the dissolved oxygen concentration remaining in the pyrene solutions after deoxygenation were performed in the same manner as the experiments previously described. An MI-730 micro oxygen-electrode (Microelectrodes, Inc.,; Londonderry, NH) an oxygen-electrode adapter (Microelectrodes, Inc.) and a pH meter (Beckman Instruments Inc.; Fullerton, CA) were used to measure the residual oxygen remaining in a 2.7×10^{-5} M pyrene solution following 0 (i.e.,solution at equilibrium with the atmosphere), 0.5, 1, 2, 3, 4, 5 and 6 minutes deoxygenation time. Variation of deoxygenation time was achieved via appropriate setting of the timer circuits (ICM 7240) of the electronic hardware. One sample from each of the twelve streams of the sampling valve was deoxygenated for each of the specified time intervals. Thus, a total of twelve samples were analyzed per each of the seven deoxygenation times. Stainless steel tubing and fittings were used throughout the system in order that deoxygenation, fluorescence analysis and measurement of residual oxygen could be achieved without back diffusion of oxygen from the atmosphere into the system. Using the data obtained in these experiments, the fluorescence intensity of the sample was correlated to the amount of oxygen present in the sample. In addition, the oxygen concentration remaining in the sample after deoxygenation was correlated to deoxygenation time. Thus, the restriction of 5 minutes deoxygenation time that was imposed on the previous

experiments was removed. From these data, the deoxygenation efficiency of the apparatus at each time interval was calculated for cyclohexane media.

Lifetime Comparison Experiments. Experiments to demonstrate the relationship between the fluorescence lifetime of various polynuclear aromatic compounds (PNA) and the enhancement factor that could be obtained via deoxygenation of the compounds were performed at 5 minute deoxygenation intervals using the PNA listed in Table I. Anthracene (99.9% purity), chrysene (98% purity), naphthalene (99% purity), and pyrene (99%+ purity) were obtained from Aldrich Chemical Company. Toluene (99.9% purity) was obtained from J.T. Baker Chemical Company. The 2,3-dimethyl naphthalene was obtained from Chem Service (West Chester, PA). The 1-methylpyrene was obtained from Molecular Probes (Eugene, OR). All PNAs were used as obtained without further purification. Each PNA was prepared in cyclohexane solution. All deoxygenation experiments were performed as described previously. Each PNA was sampled once from each stream of the sampling valve, for a total of twelve analyses per compound. These data were used to demonstrate the relationship between fluorescence lifetime of the compound and the enhancement of fluorescence intensity that can be obtained via sample deoxygenation.

Phosphorescence Experiments. In order to demonstrate the applicability of the automated deoxygenation system to room temperature phosphorescence analysis in fluid solution, 1×10^{-4}M 1-bromonaphthalene (Aldrich Chemical Company) in 1×10^{-3}M β-cyclodextrin (Advanced Separation Technologies, Inc.; Whippany, NJ) in aqueous solution was prepared. The resulting inclusion complex was deoxygenated and its phosphorescence intensities prior to and following deoxygenation were compared. From these data the usefulness of the automated deoxygenation apparatus in solution room temperature phosphorescence measurement was assessed.

RESULTS AND DISCUSSION

The reproducibility and dispersion characteristics of the deoxygenation apparatus were evaluated by repetitive injection of a 100 μL sample. Twelve injections were used to calculate an average relative standard deviation of ±3.5%. The dispersion coefficient of the apparatus was 1.5. This was determined from the ratio of the fluorescence intensities before and after dispersion. Such limited dispersion permitted maintenance of the integrity of the sample plug while achieving satisfactory sample deoxygenation. The pyrene calibration experiments were designed for two purposes: (1) to evaluate the reproducibility and precision of the deoxygenation system and (2) to establish the linear range of fluorescence emission for pyrene at fixed deoxygenation time. The reproducibility and precision was evaluated for each concentration of the pyrene solution and for the overall experimental procedure. The average relative standard deviation for all experiments was ± 3.6% of the observed fluorescence intensity. Two calibration curves over the linear range of pyrene fluorescence emission were constructed using (1) deoxygenated and (2) air equilibrated samples of the same concentration. Each curve was linear over three orders of magnitude with a slope of 5.8×10^8 and correlation coefficient of 0.999966 (deoxygenated) and slope 4.1×10^7 and correlation coefficient of 0.997 (air equilibrated). A 14-fold decrease in the detection limit of pyrene is attainable via sample deoxygenation. This is based on the ratio of the slopes of the two calibration curves, which equals the enhancement factor that can be expected for deoxygenation of the pyrene solutions.

The oxygen calibration experiments were designed for three purposes: (1) to correlate the fluorescence intensity observed in the pyrene solutions to the residual oxygen remaining after deoxygenation, (2) to correlate the residual oxygen present after deoxygenation to the amount of time used to

deoxygenate the samples and (3) to determine the deoxygenation efficiency of the apparatus for cyclohexane media at seven different intervals of deoxygenation time.

All of the previous experiments were performed using 5 minute deoxygenation times and several concentrations of analyte. Consequently, the percentages of residual oxygen remaining in all of the solutions following deoxygenation should be equal within experimental error. The differences in the fluorescence intensities of these solutions resulted solely from the differences in their concentrations. Alternatively, by using different periods of deoxygenation time and a single concentration of analyte, its fluorescence intensity could be related to the different amounts of dissolved oxygen remaining in the solution after different intervals of deoxygenation time. The results of such an experiment using 2.7×10^{-5} M pyrene and deoxygenation intervals of 0 (i.e., air equilibrated solutions), 0.5, 1, 2, 3, 4, 5 and 6 minutes were evaluated. As expected, the fluorescence intensity increased as the residual oxygen concentration and, consequently, the degree of quenching decreased. From these experiments we were also able to correlate the amount of residual oxygen remaining in the analyte to deoxygenation time. In this way, we were able to remove the restriction of 5 minutes deoxygenation time that was imposed on the previous experiments. Obviously, the dissolved oxygen concentrations and, therefore, the degree of quenching, should decrease as deoxygenation time is increased. Most importantly, however, the amount of residual oxygen, the observable fluorescence intensity and the amount of deoxygenation time that should be used for an experiment were determined. The deoxygenation efficiency of the apparatus, i.e., the ratio of the dissolved oxygen concentrations with and without deoxygenation, was also

calculated. The results are shown in Figure 4. Deoxygenation efficiency and deoxygenation time are directly related, as predicted by Fick's Law. From these data one may predict the amount of deoxygenation time required for any experiment, depending upon the degree of deoxygenation efficiency necessary for a successful experiment, or the amount of residual oxygen that can be tolerated in the cyclohexane system.

Initially, we used pyrene to demonstrate the potential of the automated deoxygenation apparatus. Because of its long fluorescence lifetime and resultant sensitivity to oxygen quenching, pyrene is an optimum compound for evaluating the usefulness of our deoxygenation apparatus. However, since not all luminescent compounds are as sensitive to oxygen quenching as is pyrene, we felt it necessary to also demonstrate the potential of the deoxygenation apparatus on compounds of varying lifetime. In this manner the relationship between lifetime and enhancement factor could be illustrated. In doing so, we selected a group of compounds with a variety of lifetimes ranging from very short to very long. These data are shown in Figure 5. For compounds that are very short-lived, such as anthracene (τ = 4.6 ns in deoxygenated cyclohexane) (23), the enhancement is correspondingly small. The enhancement factor obtained for anthracene was 1.30. Whereas, for compounds such as naphthalene that have moderate fluorescence lifetimes (i.e., 96 ns in deoxygenated cyclohexane) (23) the enhancement factors will be greater. In this study an enhancement factor of 4.7 was obtained by deoxygenation of naphthalene. Long-lived fluorescent species, examples of which were 1-methylpyrene and pyrene in this study, will be those for which sample deoxygenation will result in the greatest benefit. This will be reflected in large enhancement of fluorescence intensity and a significant decrease in detection limit.

Since room temperature phosphorescence in fluid solution is greatly hindered by oxygen quenching, sample deoxygenation plays an even more important role in phosphorescence measurement than in fluorescence measurement. In order to demonstrate the applicability of the automated deoxygenation system to phosphorescence measurement, we used the inclusion complex formed with 1-bromonaphthalene and β-cyclodextrin. Cyclodextrin complexes have been shown to be somewhat insensitive to oxygen quenching (24). Nevertheless, the phosphorescence emission could be increased by 40% of the original intensity using the automated deoxygenation apparatus. Enhancement factors comparable to those in fluorescence analysis could not be calculated. Initial levels of phosphorescence in air equilibrated solutions of the cyclodextrins is difficult to judge because shaking the solutions changes the phosphorescence emission intensity of the inclusion complex due to: (1) the participation of oxygen in equilibrium of the inclusion complex and (2) mixing of the inclusion complex which tends to precipitate and settle out of solution. However, we feel that due to the partial quenching of emission in cyclodextrin-stabilized room temperature phosphorescence and total quenching of emission in micelle-stabilized room temperature phosphorescence that occurs without deoxygenation, the usefulness of an automated deoxygenation procedure such as this is apparent. Consequently, we are currently using the procedure in both micelle-stabilized and cyclodextrin stabilized room temperature phosphorescence studies where its role is expected to be quite significant.

CONCLUSIONS

These data have been used to demonstrate the advantages that can be accrued by sample deoxygenation prior to luminescence analysis. While our procedure has been completely automated, this is not absolutely necessary for anyone wishing to use the procedure on a simple basis.

We realize that in selecting pyrene as a test compound to demonstrate our deoxygenation procedure that we have chosen one of the best possible examples. Because of its long fluorescence lifetime (i.e., 450ns in deoxygenated cyclohexane solution), pyrene is highly susceptible to oxygen quenching (25) and is, therefore, an excellent tool with which to evaluate our procedure. Nonetheless, this procedure is applicable to any fluorescent or phosphorescent species. However, the magnitude of each enhancement factor will depend on the fluorescence lifetime of the compound.

As we have also demonstrated, the deoxygenation efficiency and, thus, the magnitude of the luminescence enhancement, depends on the length of deoxygenation time. Another important factor in determining deoxygenation efficiency is the solvent used. Since molecular, gaseous oxygen is nonpolar, it becomes increasingly soluble as solvent polarity decreases (26,27). Therefore, nonpolar solvents will generally contain higher concentrations of dissolved oxygen. However, this higher oxygen concentration must also be considered in conjunction with the solvent viscosity.

Finally, while the usefulness of the deoxygenation procedure was demonstrated with applications to luminescence analysis, the procedure can be used in other modes of analysis such as high-performance liquid chromatography, reductive electrochemical analysis and liquid chromatography with electrochemical detection where problems also arise due to the presence of dissolved oxygen (28-30). Slight modifications of the deoxygenation procedure may be necessary prior to application in these fields. However, the same theory and underlying principles apply.

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CREDIT

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TABLE I. COMPOUNDS USED TO ILLUSTRATE EFFECT OF LIFETIME ON DEOXYGENATION ENHANCEMENT FACTOR

PNA	MEAN* LIFETIME (ns)	λ_{ex} / λ_{em} (nm)
anthracene	4.9	355 / 398
toluene	34	260 / 280
chrysene	44.7	320 / 381
2,3-dimethyl naphthalene	78	273 / 330
naphthalene	96	277 / 336
1-methyl pyrene	235**	342 / 396
pyrene	450	336 / 350

* Data compiled from reference 23.

** Data compiled from reference 25.

FIGURE CAPTIONS

FIGURE 1: A schematic representation of the automated deoxygenation apparatus.

FIGURE 2: A schematic representation of the dialyzer used to mount the oxygen permeable membrane.

FIGURE 3: A flowchart illustrating control of the deoxygenation apparatus.

FIGURE 4: Efficiency of the automated deoxygenation system per interval of deoxygenation time.

FIGURE 5: The effect of lifetime of PNA on the enhancement factor obtained via sample deoxygenation.

Figure 1: A schematic representation of the automated deoxygenation apparatus.

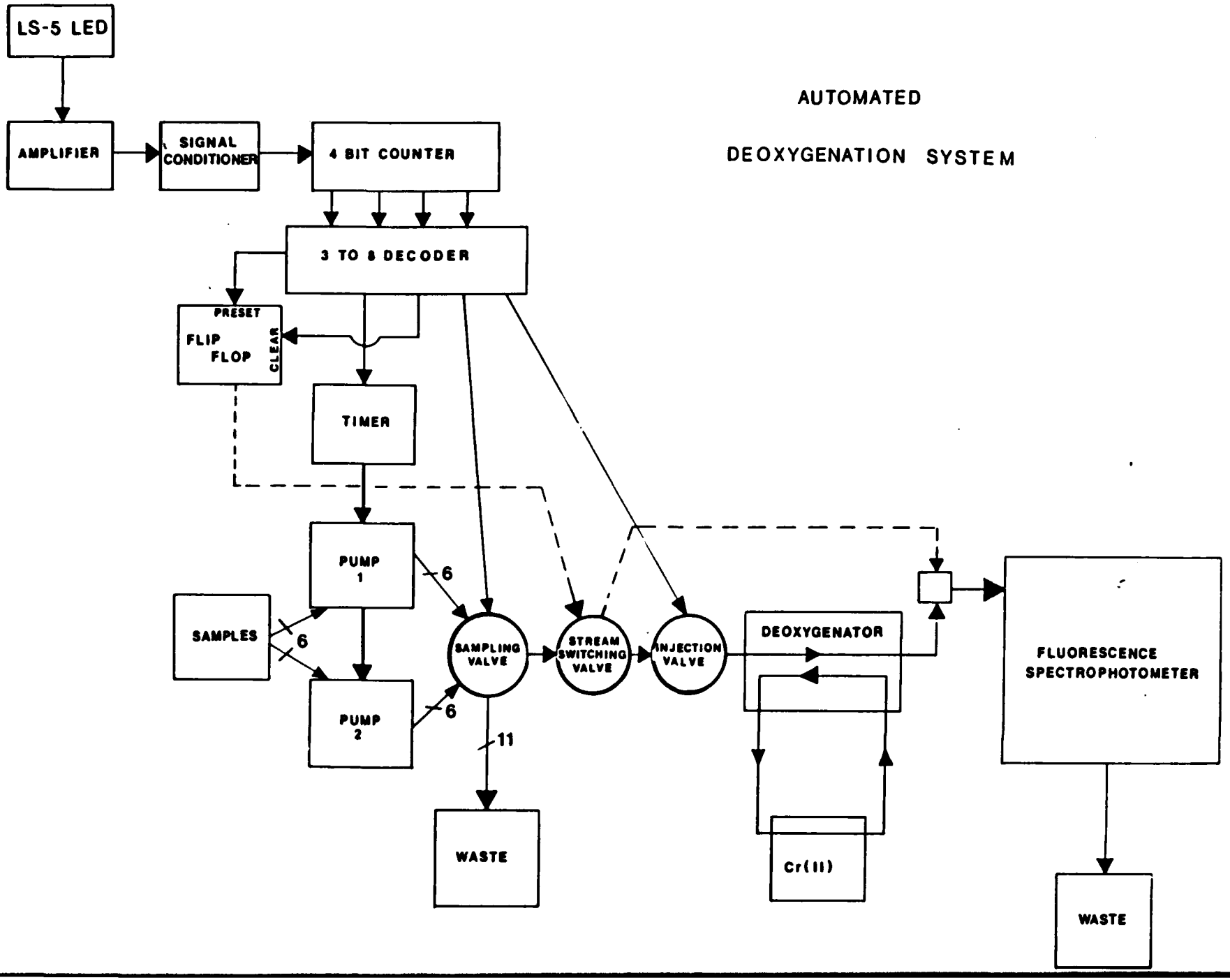
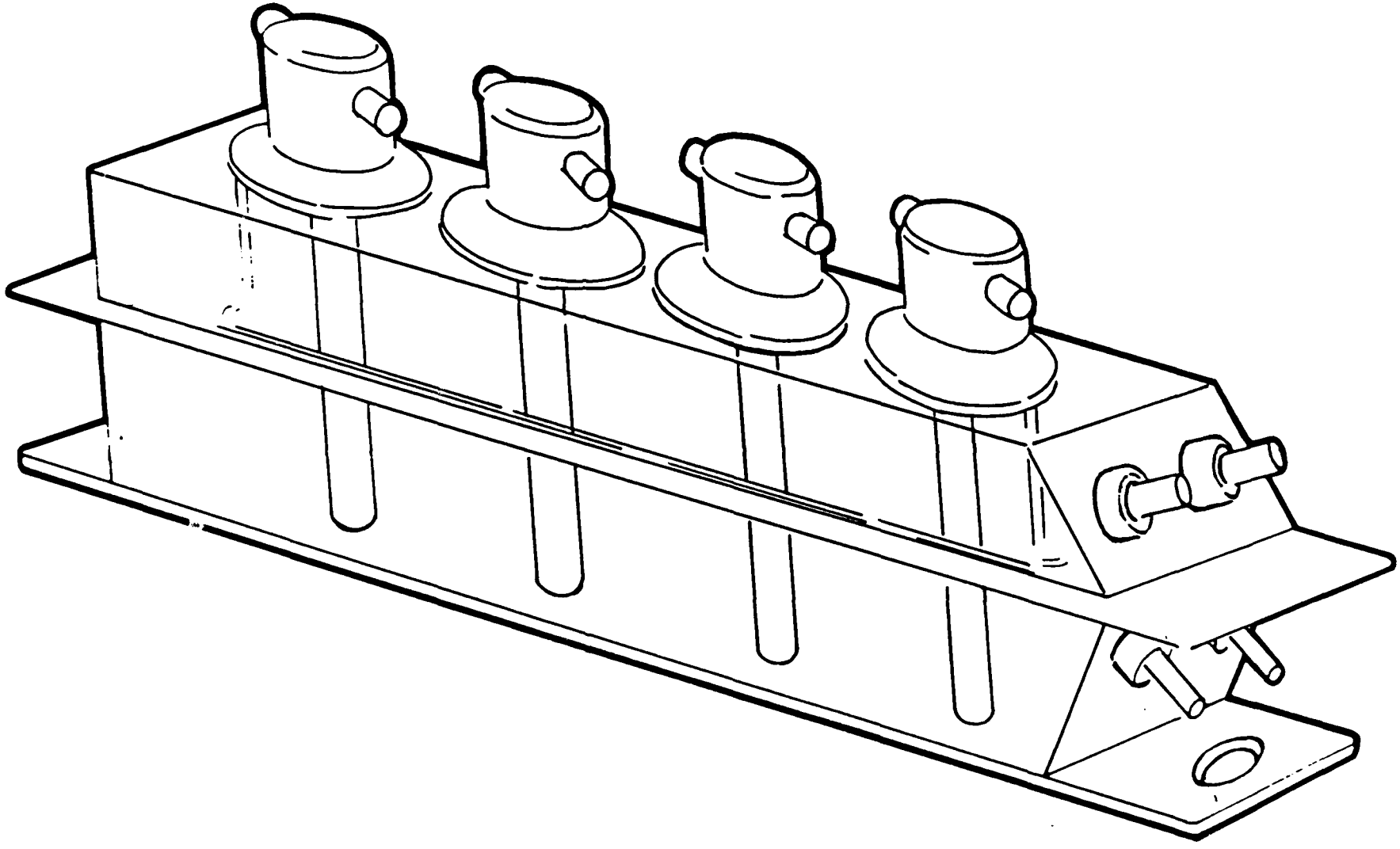


Figure 2: A schematic representation of the dialyzer used to mount the oxygen permeable membrane.



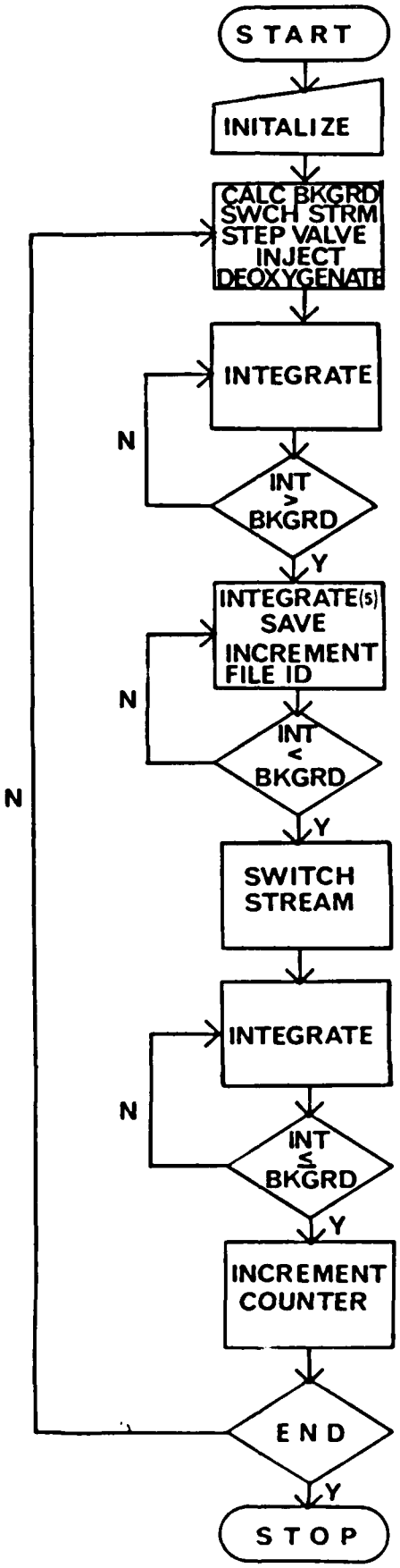


Figure 3: A flowchart illustrating control of the deoxygenation apparatus.

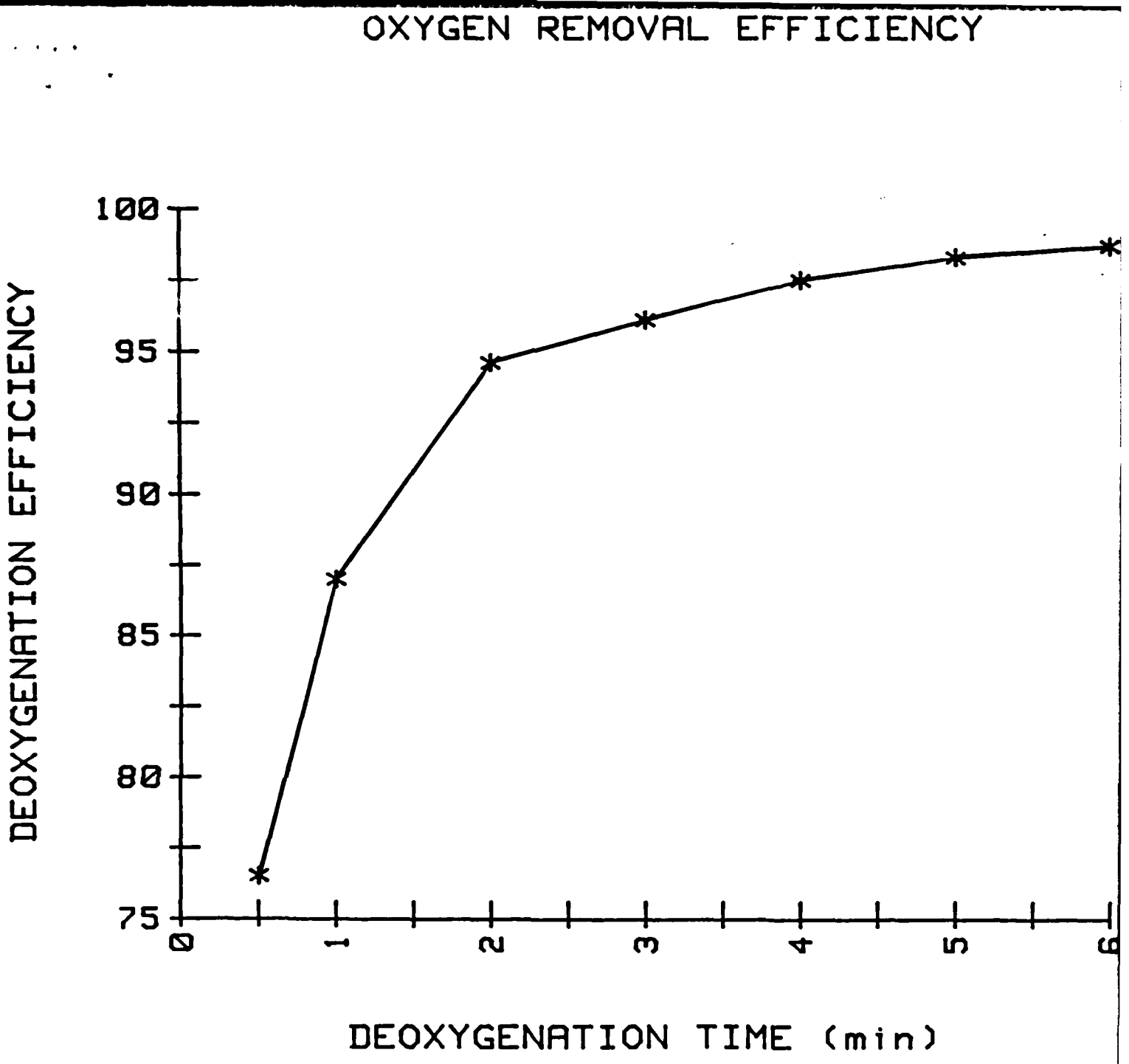


Figure 4: Efficiency of the automated deoxygenation system per interval of deoxygenation time.

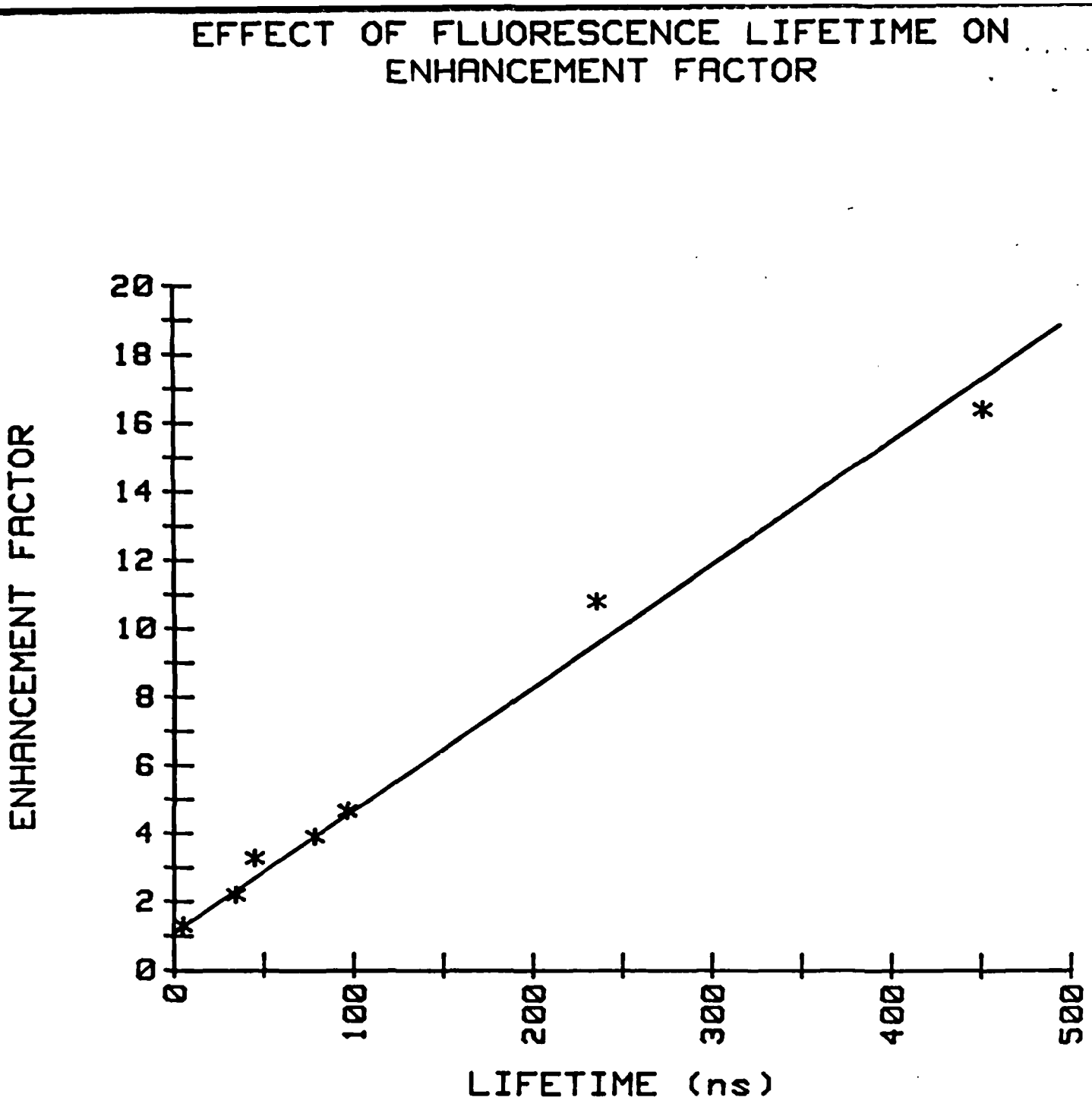


Figure 5: The effect of lifetime of PNA on the enhancement factor obtained via sample deoxygenation.

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